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CALCIUM ABSORPTION IN RATS WITH
CHOLESTYRAMINE INDUCED
STEATORRHEA

LEWIS LANDSBERG

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CALCIUM ABSORPTION IN RATS WITH
CHOLESTYRAMINE INDUCED STEATORRHEA

Submitted in partial
fulfillment of the requirement
for the degree in medicine

Lewis Landsberg

A. B. Williams College, 1960

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INTRODUCTION

The present study was undertaken to elucidate the relationship between increased fecal fat and the absorption of calcium. The clinical association of calcium deficiency with steatorrhea has been appreciated for some time. In 1919 Bassett-Smith¹ reported a case of tetany in association with sprue. Findlay and Sharpe², in the same year, described a case of celiac disease and chronic tetany in which calcium absorption was reduced to approximately one-half of normal. Barach and Murray³, adding another case to the literature, suggested that the decreased calcium absorption might be due to the physical effects of increased fecal fat. They also speculated that secretion of calcium by an irritated large bowel might be a factor in calcium loss. Ryle⁴, in 1924, postulated, that the formation of insoluble calcium fatty acid soaps might play a role in the excessive fecal calcium loss associated with steatorrhea.

In 1926 Telfer⁵ reported the results of a systematic study of calcium, phosphorous, and fat absorption. Analysis of stool samples revealed that in normal persons: (1) tryglycerides formed less than five per cent of total fecal fat; (2) calcium soaps accounted for only ten per cent of the fatty acid salts; and (3) only 20 to 30 per cent of the total fecal calcium was present as calcium soaps, the remainder being present as calcium phosphate.

In patients with steatorrhea, Telfer reported that: (1) fecal fat concentration definitely affected the excretion of calcium and phosphate; (2) impaired fat absorption was not associated with deficiency in the digestive hydrolysis of fat; and (3) the increased concentration of intraluminal fatty acids facilitated the formation of calcium soaps at the expense of calcium phosphate. Telfer also found that cod liver oil increased calcium absorption in rachitic children. These studies, then, implicated both intraluminal fat and vitamin D as factors of importance in calcium absorption.

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Linder and Harris , noting the association of fat malabsorption and tetany found that not more than one quarter of total fecal calcium could be accounted for by soaps. Although they considered that fat might physically interfere with calcium absorption, these authors concluded that failure to absorb adequate amounts of vitamin D was the chief factor. They found, indeed, that fat restriction and oral vitamin D were effective in restoring calcium absorption. The same conclusion was reached by Bauer and Marble when they succeeded in improving calcium balance in patients with osteomalacia and chronic diarrhea by the administration of oral vitamin D.

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Bassett et al. while investigating metabolism in patients with steatorrhea, found that a high fat diet increased fecal calcium loss, and conversely, that a high calcium diet impaired the absorption of

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fat. Werner and Lutwak have reported similar findings in studies with rats. These findings imply an intraluminal interaction of

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calcium and fat. Bassett and his group did note, however, that large oral doses of vitamin D improved the calcium balance in patients with steatorrhea.

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In 1948, Fourman and Spray investigated the absorption of vitamin D in steatorrhea. Finding that calcium absorption did not improve on a fat-free diet, they concluded that calcium soap formation could not entirely explain the impaired calcium absorption and osteomalacia associated with steatorrhea. The administration of vitamin D, however, restored calcium absorption to normal in the presence of a normal fat intake.

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Nicolaysen et al. in an extensive review of calcium metabolism found that as much as 500 mg. of calcium could be present in the feces in the form of soaps. Stearic acid was noted to have the greatest affinity for calcium, shorter chain fatty acids being much less effective.

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Comfort and his group at the Mayo Clinic published the results of an intensive study of eleven cases of nontropical sprue. Fecal calcium losses in these patients were greater than normal and tended to be in excess of intake. They considered several possible mechanisms for the calcium loss: decreased transit time; decreased

gastric acidity; physical effects of fat; insoluble soap formation; vitamin D deficiency; and specific mucosal defect in calcium absorption. They noted that although soap formation probably occurs, the amounts actually present could account for no more than a small proportion of fecal calcium. Recognizing that deficiencies of vitamins A and K occur in this group of patients, and that the hypocalcemia which developed was responsive to vitamin D, these authors implicated vitamin D deficiency in the pathogenesis of the calcium depletion; they pointed out, however, that the absorptive deficit in non-tropical sprue is extensive and may affect calcium independently.

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In 1959 Nassim et al. described the effect of vitamin D on nine patients with sprue. Five of these patients remained resistant to oral and parenteral vitamin D as long as they were maintained on a normal diet. On a gluten-free diet, however, responsiveness to vitamin D, with a concomitant increase in calcium absorption, returned rapidly despite persistent steatorrhea. They concluded that an actual gluten-induced resistance to vitamin D was probably an essential factor in the calcium loss associated with sprue.

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Nordin , in a study of calcium metabolism in malabsorption syndromes reported that fecal fat and fecal calcium were not directly related. He asserted that the underlying intestinal

malfunction affected calcium absorption independently.

These articles document the frequency with which steatorrhea is associated with alterations of calcium absorption. In the United States and England, steatorrhea is cited as the most common cause of osteomalacia, and an important cause of tetany as well. Calcium depletion secondary to malabsorption of fat has been explained in several different ways. The mechanisms generally invoked include: (1) non-specific physical effects of increased fecal fat; (2) formation of insoluble calcium soaps; (3) vitamin D deficiency secondary to general fat malabsorption; and (4) an intestinal mucosal defect in the calcium absorptive mechanism.

Much of the confusion regarding steatorrhea-induced calcium malabsorption is related to the use of hospital patients with steatorrhea as the subjects of investigation. These patients suffer from a variety of diseases of malabsorption which are at present not clearly distinguished, and only poorly understood. Even in groups of patients with the same diagnosis, the stage of the underlying process, the degree of steatorrhea, and the extent of calcium and vitamin D depletion vary widely from patient to patient. The present study was undertaken to clarify the nature of the relationship between fecal fat and fecal calcium under controlled laboratory conditions. The

problem was investigated by calcium balance studies performed on rats before and after the induction of steatorrhea with cholestyramine, a bile acid binding resin.

MATERIALS AND METHODS

1. Balance Studies

Balance studies were conducted on eight male albino rats of the Sprague-Dawley strain. Two litters of four rats each were studied over a two month period according to the methods of Reifens-
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stein and Albright. Control observations were made during two periods of four to seven days each, while the animals were maintained on a standard laboratory diet. The experimental diet containing cholestyramine was then introduced and the days of the study numbered from this time. After six days of experimental diet, two consecutive four day periods were analysed as described below. Following fourteen days of this diet, four of the animals were sacrificed for histologic study of the gut, and the others returned to standard laboratory chow. Supplementary metabolic studies were performed on three of the surviving rats.

The rats were maintained in metabolic cages (Acme 96B) in a constant temperature room with incandescent lighting. The metabolic cages permitted daily assessment of dietary intake and daily collection of stools without contamination by diet or urine. The stools for each day were stored in the freezer and subsequently analysed for calcium and fat as described below. The animals were weighed daily and their growth recorded.

Spot urine specimens were analysed and found to contain negligible calcium. Since urinary calcium is dependent upon the serum calcium level, and accounts for only a small percentage of daily calcium excretion^{5,18}, further analyses of urine were not performed.

Diet. The standard diet consisted of pulverized Purina laboratory chow. Distilled water was provided ad libitum in unmeasured amounts. The animals were fed once a day during the control periods and twice a day after the induction of steatorrhea. Intake was calculated after each feeding. Daily variation in intake approximated five per cent.

Samples of chow were analysed for calcium and fat by the same methods used for determination of these substances in the feces. The values obtained were used in calculating the daily intake of these nutrients.

Cholestyramine. Cholestyramine is the chloride salt of a basic anionic exchange resin which sequesters bile acids in the gut¹⁹ by the formation of an insoluble, nonabsorbable complex. In clinical trials it has been shown to lower serum cholesterol levels, to relieve the intractable pruritus of biliary cirrhosis, and, in larger doses, to produce steatorrhea.^{19,20} It is currently being²¹ investigated for use in hyperlipemic states.

Cholestyramine was first found to produce steatorrhea in humans by Hashim et al. in 1961. Fifteen grams of resin per day produced no significant increase in fecal fat, but thirty grams was associated with a four-to-five-fold increase in fat excretion. Similarly, I¹³¹ triolein was absorbed normally on the fifteen gram regimen but significantly depressed at the higher dosage. Absorption of I¹³¹ oleic acid was not impaired even at the higher dosage. These workers concluded that cholestyramine produced maldigestion of dietary fat by exclusion of the bile acids from their normal role in the hydrolysis of intraluminal intestinal fat. The resin is not known to be associated with any histologic or functional alteration of the
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bowel mucosa.

Cholestyramine for the present study was provided by the Meade Johnson Laboratories in a powdered form said to contain 0.83 grams of anhydrous resin for each gram of powder. Diets containing 2.5, 5.0 and 10.0 per cent cholestyramine were prepared by thorough mixing of the powder with the chow. These diets were administered to the animals during the experimental periods. Cholestyramine was also analysed for calcium and titratable fat by the same methods used for analysis of diet and stool.

Stool Homogenates. The stools for each metabolic period were converted into a semi-liquid alcoholic homogenate which served as

the substrate for subsequent calcium and fat determinations. The final volume of the homogenate (in milliliters) was approximately ²² six times the weight of the stool collection (in grams), and was so constituted that fifty per cent of the mixture was ethanol, forty-five ²³ per cent stool and water, and five per cent cellulose gum, the latter serving as an emulsion stabilizer. The weight of the homogenate was recorded. Since each homogenate represented the total stool collection for a particular metabolic period, the weight of the homogenate served as the basis for calculation of the calcium and fat balance.

A rounded spoonful of silica rocks was added to the weighed mixture which was then homogenized by vigorous shaking for one hour in an automatic paint shaker. The homogenate was stored in the freezer until needed, at which time it was reshaken and allowed to equilibrate with room temperature before use.

2. Calcium Analysis

Duplicate weighed aliquots of each homogenate were dried, ashed, extracted with dilute HCL, and the calcium content of the ²⁴ extract determined colorimetrically by the method of Roe and Kahn.

Preparation of the Extract. Each weighed sample of homogenate was dried in a crucible at 60 degrees C and then transferred to a

muffle oven and ashed at 600-700 degrees C for sixteen hours.

The ash was crushed with a tamper, extracted four times with warm,
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ten per cent HCl and diluted up to 500 ml. with distilled water.

Calcium Determination. Duplicate aliquots of each ashed
extract were assayed for calcium by a modified version of the Roe
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and Kahn serum calcium method. In adapting this procedure to the
protein-free ashed extract, it was found that precipitation with
trichloroacetic acid and subsequent filtration could be eliminated.
This extract was alkalinized, precipitated with tribasic sodium
phosphate, and the calcium phosphate precipitate washed with an
alkaline mixture of ethyl and amyl alcohols. This precipitate was
dissolved in a solution of ammonium molybdate in H_2SO_4 , with the
formation of phospho-molybdate. The phosphate was determined
colorimetrically in a Klett photoelectric colorimeter, after the
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addition of aminonaphtholsulfonic acid. The calcium content was
calculated from the concentration of phosphate present.

Recovery of Weighed Samples of $CaCl_2$. Two samples of
calcium chloride were weighed out, dissolved in 10 per cent HCl
and determined in the same manner as the extract of ashed homo-
genate. Recovery of calcium by this method was 95.8 and 97.1
per cent respectively. (Table I).

TABLE I RECOVERY OF CaCl₂

Sample	Wt. of CaCl ₂ (g)	Calcium content (g)	Calcium Det'd by method (g)	Recovery %
1	1.4331	0.5164	0.4948	95.8
2	0.6770	0.2440	0.2377	97.1

The Effect of Magnesium. Magnesium in large amounts is said to precipitate phosphate, and thus be erroneously determined as calcium by the present method²⁴. A sample of magnesium sulphate was extracted and determined in the same fashion as the extract of ashed homogenate. One gram of magnesium was found equivalent to 20.8 mg. of calcium. This was considered a negligible contribution to fecal calcium in view of the minute quantities of magnesium contained in the diet.

3. Fat Analysis

The fat content of the homogenate was determined titrimetrically^{23, 27} after solution of the lipids in alcohol and extraction into toluene.²⁸ Van de Kamer et al.²⁸, in 1949 were the first to describe a suitable volumetric analysis for fecal fat. Jover and Gordon²³, in 1962, modified Van de Kamer's method by extracting the lipids into toluene rather than petroleum ether, thereby increasing the recovery

of hydroxy acids and improving the yield of total fat.

Total Fecal Fat. Duplicate weighed 20 ml. aliquots of homogenate were saponified by refluxing with 5 ml. of 66 per cent KOH and 40 ml. of 95 per cent ethanol for 20 minutes. The samples were then acidified with 17 ml. of 25 per cent HCl, and extracted with 50 ml. of toluene. Twenty-five ml. of the toluene phase was subsequently evaporated, the residue resuspended in 10 ml. of neutral ethanol and the fatty acid titrated with 0.1N NaOH to a thymol blue endpoint. Total fat was calculated as milliequivalents of titratable fatty acid, with correction for the incomplete extraction of lipids into toluene, and the expansion of the toluene phase as described²³ by Jover and Gordon²³. The correction factor was 1.15. In conversion of milliequivalents of fatty acid to milligrams of fat^{23, 29} an average molecular weight of 284 was assumed.

Fractionation of Fecal Fat. Saponification and subsequent acidification of the stool homogenate in the above method liberate all titratable lipid and hence provide an estimate of total fecal fat. Estimation of the amount of neutral fat, free fatty acids, and soaps was attempted by modifying these procedures. By eliminating both saponification and acidification, only that portion of fecal fat present as free fatty acid is titrated. Alternatively, acidifying in the absence of hydrolysis converts the soaps to free fatty acids and

the titre in this case includes both free fatty acids and soaps. This fractionation scheme is outlined in figure I.

Figure I. Titrimetric Fractionation of Fecal Fat Stool Homogenate

Procedure	Extraction	Acidification Extraction	Saponification Acidification Extraction
Lipids Extracted	Neutral fats	Soaps	Soaps
	Free Fatty Acids	Neutral fats	Neutral fats
	Fatty acid Esters	Free fatty Acids	Free fatty Acids
	Non-titratable Lipids	Fatty Acid Esters	Fatty Acid Esters
		Non-titratable Lipids	Non-titratable Lipids
Lipids Titrated	Free Fatty Acids	Soaps	Soaps
		Free Fatty Acids	Free Fatty Acids
			Fatty Acid Esters
			Neutral Fats
			{Total titratable fat}

Free Fatty Acids. Duplicate weighed 50 ml. aliquots of homogenate were mixed with 20 ml. of 60 per cent ethanol and

10 ml. of 95 per cent ethanol. This mixture of alcohols provided a final ethanol concentration of 60 per cent thus assuring maximal
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extraction into toluene . The mixture was then extracted, evaporated, resuspended and titrated in the manner described above.

Soaps. Duplicate weighed 30 ml. aliquots of homogenate were acidified with 10 ml. of 25 per cent HCl thereby providing the same acidity as in the total fat determination. Heating the homogenate with acid was found to definitely decrease the fatty acid yield (presumably by facilitating esterification) and was thus avoided. After the addition of 25 ml. of 60 per cent ethanol and 20 ml. of 95 per cent ethanol, extraction, evaporation, and titration were performed as described above.

Neutral Fat. Neutral fat was calculated by subtracting the sum of the free fatty acids and the soaps from the total fat.

4. Histology

Half of the animals were sacrificed at the conclusion of the two week cholestyramine regime for histologic study of the intestinal mucosa. Animals on each dosage schedule were included along with several control animals on standard diets. The animals were exsanguinated, and the gut irrigated with saline and formalin. Specimens of duodenum, jejunum, terminal ileum, and liver were

sectioned and studied with trichrome, mucoprotein and hematoxylin and eosin stains.

RESULTS

1. Analysis of Diet

Chow. The stated calcium content of Purina laboratory chow is 1.42 per cent by weight (method unspecified). Using the colorimetric method of Roe and Kahn, as described above, the chow was found to contain 1.46 per cent calcium. This value was used in the calculation of calcium balance.

The fat content of chow as determined by the modified Van de Kamer method was 4.8 per cent by weight. Similarly, this value was used in calculating the extent of steatorrhea during the metabolic periods. Fractionation of the fat content of the chow revealed that neutral fat constituted 2.5 per cent, free fatty acids 2.2 per cent, and soaps 0.1 per cent, respectively.

Cholestyramine. Analysis of samples of cholestyramine by the same methods revealed the resin to be a negligible source of dietary calcium or fat. Accordingly, cholestyramine was ignored in calculating the intake of these substances.

2. Balance Studies

Every animal gained weight throughout the period of study. Growth curves are represented in figures 4 through 11. Although some of the animals gained less during the steatorrhea period, at no time did growth cease. Failure to lose weight in the presence

of massive steatorrhea was probably secondary to an increased dietary intake. Thus, while the mean intake for all eight rats was 20.4 grams per day during the control periods it rose to 24.0 grams per day during steatorrhea, an increase of about 20 per cent.

Of the two litters of animals, the first was older and larger. At the outset of the control period the rats in the first litter weighed 135 to 160 grams while those of the second litter weighed 120 to 135 grams. Similarly, at the inception of cholestyramine administration, the weights were 205 to 230 for the first litter and 185 to 205 for the second. Because of the difference in age and size, the data for the two groups of animals is plotted separately.

Steatorrhea. All rats receiving the cholestyramine regimen developed steatorrhea. The degree of steatorrhea was expressed as a percentage of dietary fat appearing in the feces:

$$\text{Per cent steatorrhea} = \frac{\text{fecal fat (g/d)}}{\text{dietary fat (g/d)}} \times 100$$

This method of representation is commonly used as a convenient parameter of steatorrhea, and is not meant to imply the identity of fecal and dietary fats.

During the control periods the mean fecal fat excretion was 18 per cent of the dietary fat for all eight animals, with no difference between the litters. This was taken as a measure of

normal fecal fat excretion in these rats. The animals on the 2.5 per cent cholestyramine diet had a mean steatorrhea of 37.1 per cent; those receiving 5.0 per cent cholestyramine averaged 57.2 per cent steatorrhea; those on the 10.0 per cent cholestyramine diet excreted an average of 69.1 per cent of the dietary fat in the feces. The mean values for each regimen appear in table II; values for the individual metabolic periods are recorded in tables IV and V. The close relationship between the gram intake of cholestyramine and the degree of steatorrhea is shown in figure 2.

Fecal fats were fractionated for four control and five experimental periods (table III). In the control periods 39 per cent of total fecal fat was present as neutral fats, 13 per cent as soaps, and 48 per cent in the form of free fatty acids. During steatorrhea the free fatty acid fraction did not change appreciably, but neutral fats decreased sharply with a concomitant increase in soaps. This direct relationship between the degree of steatorrhea and the soap content of the stools is indicated in figure 3.

Calcium. Calcium balance for each metabolic period was expressed as follows:

$$\text{Per cent absorption calcium} = \frac{\text{dietary ca. (g/d)} - \text{fecal ca. (g/d)}}{\text{dietary ca. (g/d)}} \times 100$$

This provided a measure of the net absorption of dietary calcium.

The relationship between calcium absorption during the experimental periods, and calcium absorption during the control periods, for each animal, was represented as an index in the following way:

$$\text{Index of calcium absorption} = \frac{\text{per cent absorption ca. experimental period}}{\text{per cent absorption ca. control period}}$$

Hence, an index of greater than 1.0 indicates calcium absorption in excess of the control period. The balances for each rat are plotted graphically in figures 4-11. The data appears in tables IV and V.

Calcium absorption indices are summarized in table VI.

In the first litter, calcium absorption during the control periods varied between 20.6 and 23.8 per cent. During the first steatorrhea period, 6 to 10 days after the initiation of the cholestyramine diet, every animal showed an increase in calcium absorption by more than 50 per cent. Despite steatorrhea ranging from 50 to 75 per cent, calcium absorption indices for the period were 1.55 to 1.60. During the second steatorrhea period, days 11 to 14, two of the four animals (rats 1 and 2) continued to show increased calcium absorption with indices of 1.52 and 1.20, while the other two rats (3 and 4) showed a marked fall in calcium absorption to approximately 20 per cent of normal, with indices of 0.16 and 0.24 respectively. In three of the four animals steatorrhea further increased during the second experimental period; one animal (rat 2)

had essentially the same high level throughout both experimental periods (Table IV and VI; figures 4-7).

Two weeks after the initiation of the cholestyramine regimen two animals were sacrificed for histologic study, and two were returned to normal diet for analysis of additional metabolic periods during the recovery phase. Rat 2 had three recovery periods analysed and was the most extensively studied animal. As shown in Tables IV and VI, rat 2 failed to show decreased calcium absorption in either experimental period although the steatorrhea values for both these periods were the highest of the study (75 per cent). After return to normal diet, however, and regression of the steatorrhea to virtually normal levels, the calcium absorption fell below normal for the first time. In fact, the lowest period of calcium absorption was on days 21 through 25, five days after return to a normal diet (figure 5). On days 15 through 20 the calcium absorption index was 0.83, on 21 through 25 it was 0.34 and on days 26 through 29 it rose to 0.55. Rat 2 thus showed decreased calcium absorption in the presence of normal fecal fat following a period of normal calcium absorption associated with massive steatorrhea.

One recovery period was also analysed on rat 4. This animal had its lowest calcium absorption (24 per cent of normal) during the second steatorrhea period (days 11 to 14). Five days after the

resumption of normal diet (days 21 to 25) the absorption increased to 81 per cent of normal.

In the second litter calcium absorption during the control period varied between 31.4 and 34.9 per cent. This was appreciably higher than the corresponding values for the first litter. During the first steatorrhea period, days 6 to 10, one of the animals showed essentially normal calcium absorption, two of the animals showed minimally depressed absorption with indices of 0.82 and 0.89 and one animal showed moderate reduction of calcium absorption with an index of 0.63. There was no correlation between degree of steatorrhea and calcium malabsorption as rat 8, the only animal in this litter showing normal calcium absorption, had the most steatorrhea.

During the second steatorrhea period, days 11 to 14, one rat maintained its calcium absorption at near normal values, two rats showed moderate reduction in absorption and one rat suffered severe depression with an absorption index of 0.06. Again, there was no correlation between fat and calcium malabsorption, for the animal (rat 6) with the severely depressed calcium absorption had the least steatorrhea. The degree of steatorrhea was the same in both experimental periods (Tables V, VI; figures 8-11).

As with the first litter, two of the animals were sacrificed

after two weeks of the cholestyramine diet. One additional recovery period was analysed on rat 8. This animal had a calcium index of 1.03 during days 6 to 10 associated with 70 per cent steatorrhea, an index of 0.92 for days 11 to 14 with 57 per cent steatorrhea, and an index of 0.43 during days 15-19 after resumption of normal diet and reversion of fecal fat to normal (figure 11). Thus, like rat 2 of the first litter, this animal did not develop impaired calcium absorption until after the cholestyramine had been discontinued and fecal fat had returned to normal levels.

In all, three of the eight animals (rats 1, 2, and 8) did not show decreased calcium absorption during the steatorrhea periods. Of these three, two (rats 2 and 8) subsequently developed impaired absorption while on normal diet in the recovery period, and one (rat 1) was sacrificed immediately after the second steatorrhea period without further study. Thus, every animal but this one (rat 1) showed decreased absorption during or after the induction of steatorrhea. It is apparent, however, from tables IV and V and figures 4-11 that there was no correlation between the extent of the steatorrhea and the decrease in calcium absorption. There was, rather, a latent period of variable length between the onset of steatorrhea and the development of calcium malabsorption. After six days of steatorrhea one animal had decreased calcium absorption;

after eleven days four more animals showed calcium malabsorption; the other two rats showed significant impairment of calcium absorption 12 and 21 days after the onset of steatorrhea, at a time when fecal fat had returned to normal.

Stoichiometric Relationships. In order to evaluate the role of calcium sequestration by fatty acids in the form of soaps, daily fat and calcium excretion for each metabolic period were calculated as milliequivalents, and recorded in Tables VII and VIII. There was no stoichiometric relationship between fecal calcium and fecal fat in any metabolic period; calcium was lost in relatively huge excess of fat in both the control and steatorrhea periods. Even in the presence of massive steatorrhea fecal calcium exceeded total fecal fat by four-to-five-fold on an equivalent basis. In addition, the total fat included a substantial quantity of neutral fat (Table III) which presumably was unavailable for combination with calcium. The stoichiometric imbalance is even more marked when that portion of fecal fats present as soaps is considered. The excretion of soaps, where these were determined, is recorded in Tables VII and VIII as milliequivalents of fatty acids.

Fecal Composition. Stool weight and composition for each metabolic period are recorded in Tables VII and VIII. Stool composition was expressed as per cent fat and per cent calcium

by weight. In contrast to fecal fat, fecal calcium varied inversely with the stool weight: the greater the bulk of stool, the greater the percentage fat and the smaller the percentage calcium. This is represented graphically in figure 12.

3. Histology

Four of the eight animals were sacrificed on day 15 immediately after the second steatorrhea period. Histologic sections of the gut were compared with normal controls, and failed to reveal any abnormalities in the cholestyramine treated group. Histologic sections of the livers of two of the animals were also within normal limits.

DISCUSSION

1. Cholestyramine-Induced Steatorrhea

Cholestyramine proved to be an extremely useful agent in producing steatorrhea. By varying the dosage of resin it was possible to produce steatorrhea of graded severity without overt damage to the intestinal mucosa (Table II; figure 2).

It is of further interest that increasing degrees of steatorrhea were associated with an increase in the soap fraction at the expense of neutral fat (Table III; figure 3). This may indicate that cholestyramine, presumably through its binding of the bile acids, interferes with the absorption of hydrolyzed fats more significantly than it does with fat digestion. This is contrary to the findings of Hashim ¹⁹ et al. with I¹³¹ labelled triolein and oleic acid. It is, however, ³⁰ consistent with the findings of Isselbacher that bile salts stimulate fatty acid absorption.

2. Calcium Absorption and Steatorrhea

Differences Between the Two Litters. Analysis of the balance study data justified separate consideration of the two litters of rats on the basis of age and size. The older animals absorbed between 20.6 and 23.8 per cent of their dietary calcium during the control periods while the younger ones absorbed between 31.8 and 34.9 per cent, despite the fact that control fecal fat values were identical

for both groups. Each litter was thus a distinct but consistent group with regard to calcium absorption. The younger animals, presumably with greater need for calcium, absorbed approximately one-third more dietary calcium than the older ones. Indeed,³¹ Schacter et al. have shown that everted rat gut sacs of younger animals incorporate radio-calcium more rapidly than similar preparations from older animals.

Periods of Increased Calcium Absorption. With the onset of steatorrhea, each animal in the older litter displayed a 55 to 65 per cent increase in calcium absorption (Table IV, VI). This impressive increase in positive calcium balance during the first experimental period, occurring in association with marked steatorrhea, may be related to the increased calcium intake in this^{32, 33} period (Table IV). Lutwak and Whedon have established that an increased dietary intake of calcium may result in a prominent increase in positive calcium balance.

The younger animals did not display a similar increase in calcium absorption despite a similar increase in dietary intake (Table V, VI). These animals, however, absorbed considerably more calcium during the control periods than their fellows from the older litter. It is possible that they were absorbing calcium at a maximal rate at the start of the study and unable to further

increase this rate with an increase in intake. In addition, being more avid for calcium, these animals may have been more susceptible to the development of calcium malabsorption.

Periods of Decreased Calcium Absorption. It is apparent from tables IV, V and VI as well as figures 4 through 11 that there is no simple or direct relationship between fecal fat and fecal calcium loss. While seven of the eight animals did develop impaired calcium absorption during the study, in two of these the impairment came only after fecal fat had reverted to normal, and in five normal or increased calcium absorption coexisted with massive steatorrhea during at least one metabolic period. If the presence of fecal fat per se were the significant factor in inducing calcium loss there should have been a direct relationship between fecal fat and decreased calcium absorption at all points in the study. Therefore, the variation in calcium absorption must be explained on another basis.

The decreases in calcium absorption which occurred were both abrupt in onset and marked in extent. After the low point in calcium absorption had been reached, two of the animals (rats 2 and 4), showed subsequent rises in absorption toward normal (figures 5 and 7). These factors indicate that the impairment in calcium absorption was rather acute and presumably reversible; they further demonstrate that the aging of the animals over the

course of the study was not a sufficient cause for the decrease in calcium absorption.

The latent period, previously described, between the induction of steatorrhea and the development of calcium malabsorption, implicates a time dependent process. It is of interest in this regard that the standard bioassay for vitamin D³⁴ requires a rachitogenic diet (free of vitamin D) for a minimum of 18 to 21 days. In the present study, all surviving rats had developed calcium malabsorption by the end of 21 days from the onset of steatorrhea. This observation seems most consistent with the induction of vitamin D loss with fecal fats, and the subsequent development of increased fecal calcium loss. The fact that impaired calcium absorption became apparent after reversion of fecal fat to normal in two of the animals may be explained by the delay in restoration of depleted tissue stores of vitamin D, even after active fecal loss of the vitamin had ceased.

In order to eliminate the transition period between normal absorption and steatorrhea, and to insure stable conditions during the experimental regimen, the first six days on cholestyramine were omitted from the analysis. The two subsequent four-day intervals were selected for detailed study of calcium absorption. This selection proved to be fortuitous. Had less of a transition

period been allowed, and metabolic periods analysed after four and eight days for example, the decreased absorption of calcium might have been missed altogether. Conversely, a longer transition period would have eliminated the initial period of increased absorption and obscured an important clue to the pathogenesis of the observed calcium malabsorption. Some of the contradictory clinical studies may involve such factors.

3. Theories of Calcium Malabsorption in Steatorrhea.

Increase in Stool Volume. The suggestion has often been made that an increased stool volume in some way traps calcium and causes excessive calcium loss in the feces. The factors involved are not clearly specified, but chelation, and the nonspecific physical effects of fat globules, are often cited. The present study provides no support for this hypothesis. As shown in figure 12 and Table VII and VIII the concentration of calcium actually fell with an increase in fecal weight.

Insoluble Soap Formation. The formation of insoluble calcium fatty acid salts has been widely accepted as the basic factor producing calcium malabsorption in the presence of steatorrhea. The present data also fails to support this theory. The soap theory predicts an obligate calcium excretion in direct proportion

to fecal fat; it is clear from Table IV and V and figures 4-11 that this was not the case. Further, on a stoichiometric basis, fecal calcium exceeded fecal fat by over five-fold (Table VII and VIII).⁷ This is in accord with the findings of Linder and Harris⁷ that only one-quarter of the fecal calcium was present as soaps.

Mucosal Defect. It has recently been proposed that the impaired calcium absorption in steatorrhea results from an underlying defect in the intestinal mucosa¹⁵. Whether the supposed defect is a primary part of the malabsorption syndrome or a secondary toxic effect of the increased fecal fat, is not clear. This theory is difficult to rule out on theoretical grounds, but there is very little direct evidence in its favor.

Although it is possible that either the steatorrhea or the cholestyramine directly impaired the calcium absorptive mechanism in the animals studied here, several factors argue against this hypothesis:

(1) Direct toxic insults are often associated with histologic damage. In sprue, for example, gluten toxicity is reflected in marked mucosal change as well as the characteristic functional impairment. The intestinal mucosa was normal at the height of steatorrhea in the animals studied here.

(2) The onset of functional impairment following contact with

an offensive agent is usually rapid. In the gluten analogy, malabsorption occurs rapidly after introduction of the toxic substance into the diet. Impaired calcium absorption in the present study occurred only after a latent period of 6 to 21 days.

(3) Since the absorption of fat occurs distal to the site of absorption of calcium in the rat intestine, the concentration of fat at the site of the calcium absorptive mechanism should be the same in both normal animals and those with steatorrhea. Under these circumstances, the fat itself could hardly be implicated as the toxic factor. The direct effect of cholestyramine on calcium absorption is not known, but calcium malabsorption was not related to the dosage of cholestyramine ingested (Tables IV, V).

While these factors weigh against an organic intestinal lesion in the pathogenesis of calcium loss, they do not exclude the possibility of a functional impairment in calcium absorption. It has, moreover, been well established that vitamin D is necessary for the normal
35, 36, 37, 38
function of the calcium absorptive mechanism . Schacter
36, 37
and his group have amply demonstrated that everted intestinal sacs from vitamin D deficient rats fail to incorporate radio-calcium at a normal rate. While a functional change in the mucosa may have produced the impairment in calcium absorption in the present study, the likely defect is vitamin D deficiency.

Vitamin D Deficiency. The malabsorption of calcium demonstrated in this study is most compatible with the development of vitamin D deficiency. Steatorrhea is known to be associated with deficient absorption of the fat soluble vitamins, and, as emphasized above, vitamin D is essential for the normal absorption of calcium. Other factors, alluded to above, include: (1) The presence of a latent period, of variable but appropriate length, between the induction of steatorrhea and the development of calcium malabsorption; (2) The absence of demonstrable damage to the gut in the presence of impaired calcium absorption; and (3) The lack of correlation between calcium loss and fecal fat or fecal mass. It is also of interest that while the absorption of calcium is an active process^{31, 36}, the absorption of vitamin D is by passive diffusion³⁹, and hence more susceptible to depletion in the fecal fat.

The evidence presented here in favor of the vitamin D hypothesis is admittedly circumstantial and presumptive. It does, however, invite further investigation along these lines. Clearly, the unequivocal demonstration that vitamin D deficiency underlies the calcium loss in steatorrhea demands the reversal of impaired calcium absorption, by parenteral vitamin D, in the presence of continued steatorrhea.

SUMMARY

1. Calcium balance studies were conducted on eight rats before and after the induction of steatorrhea.
2. Cholestyramine, a bile acid binding resin, produced steatorrhea in direct proportion to the dose administered. There was no associated damage to the intestinal mucosa.
3. Seven of eight animals developed impaired calcium absorption after the induction of steatorrhea.
4. There was no relationship between the degree of steatorrhea and fecal calcium loss. Normal or increased calcium absorption coexisted with massive steatorrhea during eight metabolic periods.
5. A latent period between the induction of steatorrhea and the development of calcium malabsorption was present in every case. Two animals did not develop calcium loss until after withdrawal of cholestyramine and reversion of fecal fat to normal levels.
6. The data fail to support the calcium soap formation theory. No stoichiometric relationship was found between fecal calcium and fecal fat. Increase in fecal weight was actually associated with a decrease in fecal calcium concentration.
7. The data are most compatible with the development of vitamin D deficiency during steatorrhea. On the basis of the

present study, however, vitamin D cannot be unequivocally implicated as the critical factor in the calcium malabsorption associated with experimental steatorrhea.

TABLE II
CHOLESTYRAMINE INTAKE AND STEATORRHEA

Cholestyramine Intake		Steatorrhea (mean) %
% of diet	grams per day (mean)	
0	0	18.1
2.5	0.61	37.1
5.0	1.30	57.3
10.0	2.55	69.1

TABLE III
FRACTIONATION OF FECAL FAT

Rat No.	Dietary Choles. %	Steator- rhea %	Fractionation		
			Neutral fat %*	Soaps %*	Free fatty acids %*
I	0	16.1	40.9	8.8	50.3
I	0	17.8	38.3	15.3	46.5
3	0	18.2	46.6	7.7	45.7
6	0	17.4	33.7	15.9	50.6
7	0	17.7	33.8	18.1	48.1
MEAN CONTROL		17.4	38.6	13.2	48.2
5	2.5	39.9	26.0	21.9	52.2
6	2.5	33.1	22.6	11.3	66.1
MEAN	2.5	37.0	24.3	16.6	59.1
3	5.0	64.2	19.1	41.2	39.7
7	5.0	54.0	13.4	36.7	49.9
MEAN	5.0	59.1	16.2	38.9	44.8
I	10.0	58.1	18.1	35.4	46.5
I	10.0	68.8	20.3	42.0	37.6
MEAN	10.0	63.4	19.4	38.7	42.0

* per cent of total fecal fat

TABLE IV

BALANCE STUDY DATA-LITTER 1

Rat	Days After Onset Choles.	Intake		Fat		Steat-orrhea %	Calcium		Calcium Absorp. %	Calcium Balance Index
		Choles. g/d	Chow g/d	Diet mg/d	Feces mg/d		Diet mg/d	Feces mg/d		
1	C	0	19.00	912	154	16.9	278	212	23.8	1.00
	6-10	2184	25.55	1227	712	58.1	373	231	38.0	1.60
	11-14	2.63	23.75	1140	784	56.8	347	221	36.2	1.52
2	C	0	18.05	867	174	20.0	264	204	22.7	1.00
	6-10	2.54	22.91	1100	814	74.0	334	213	36.2	1.59
	11-14	2.18	19.59	940	709	75.4	286	208	27.3	1.20
	15-20*	0	22.87	1098	250	22.8	334	271	18.9	0.83
	21-25*	0	19.90	955	208	21.8	291	268	7.8	0.34
	26-29*	0	19.18	921	210	22.8	280	245	12.5	0.55
3	C	0	21.31	1022	186	18.2	310	246	20.6	1.00
	6-10	1.27	24.18	1161	596	51.3	353	233	34.1	1.65
	11-14	1.18	22.45	1078	693	64.2	328	317	3.3	0.16
4	C	0	19.38	929	172	18.6	283	217	23.3	1.00
	6-10	1.20	22.87	1098	545	49.7	334	213	36.2	1.55
	11-14	1.17	22.30	1070	632	59.0	326	307	5.6	0.24
	21-25*	0	21.81	1046	182	17.4	318	259	18.7	0.80

C=control period before cholestyramine begun (mean).

*-=recovery period after cholestyramine withdrawn.

TABLE V

BALANCE STUDY DATA-LITTER 2

Rat	Days After Onset Choles.	Intake		Fat		Steat-orrhea %	Calcium		Calcium Absorp. %	Calcium Balance Index
		Choles. g/d	Chow g/d	Diet mg/d	Feces mg/d		Diet mg/d	Feces mg/d		
5	C	0	19.62	943	185	19.6	286	187	34.9	1.00
	6-10	0.62	24.16	1160	477	41.1	353	251	28.7	0.82
	11-14	0.60	23.24	1116	445	39.9	339	271	20.0	0.57
6	C	0	20.24	971	164	17.4	296	203	31.4	1.00
	6-10	0.62	24.06	1155	394	34.2	351	254	27.8	0.89
	11-14	0.59	23.13	1110	367	33.1	338	332	1.8	0.06
7	C	0	22.19	1065	189	17.7	324	221	31.8	1.00
	6-10	1.36	25.68	1233	651	52.8	375	297	20.9	0.63
	11-14	1.42	26.92	1292	699	54.0	393	337	14.3	0.45
8	C	0	23.15	1111	199	17.9	338	221	34.6	1.00
	6-10	1.44	27.28	1309	923	70.5	398	156	35.6	1.03
	11-14	1.38	26.16	1256	718	57.2	382	260	31.9	0.92
	16-19*	0	26.52	1273	276	21.7	387	330	14.8	0.43

C=control period before cholestyramine begun (mean).

*=recovery period after cholestyramine withdrawn.

TABLE VI
CALCIUM ABSORPTION INDICES

Litter	Rat	Control Values Calcium Absorp- tion %	Steatorrhea		Recovery		
			Days after onset	cholestyramine			
			6-10	11-14	15-20	21-25	26-29
I	1	23.8	1.60*	1.52			
	2	22.7	1.59	1.20	0.83	0.34	0.55
	3	20.6	1.65	0.16			
	4	23.3	1.55	0.24		0.80	
II	5	34.9	0.82	0.57			
	6	31.4	0.89	0.06			
	7	31.8	0.63	0.45			
	8	34.6	1.03	0.92	0.43		

* These numbers represent calcium absorption indices, as defined in the text.

TABLE VII
STOOL COMPOSITION-LITTER I

Rat	Diet Choles. %	Wt. of Stool (g/d)	Calcium % (by wt.)	Fat % (by wt.)	Soaps (mEq/d)	Total Fat (mEq/d)	Calcium (mEq/d)
1	0*	6.70	2.96	2.06	0.043	0.485	10.32
1	0*	9.46	2.30	1.86	0.095	0.620	10.83
2	0*	6.02	3.31	-	-	-	9.96
2	0*	7.94	2.66	2.36	-	0.658	10.50
3	0*	8.20	2.76	-	-	-	11.70
3	0*	10.40	2.51	1.92	0.054	0.705	13.10
4	0*	6.83	3.14	-	-	-	10.68
4	0*	8.24	2.68	2.08	-	0.603	11.02
3	5.0	13.10	1.78	4.55	-	2.098	11.60
3	5.0	14.90	2.12	4.65	1.005	2.439	15.82
4	5.0	13.80	1.54	3.95	-	1.920	10.62
4	5.0	15.45	1.97	4.09	-	2.224	15.34
1	10.0	19.80	1.17	3.60	0.887	2.508	11.54
1	10.0	19.05	1.16	4.11	1.161	2.762	11.04
2	10.0	17.20	1.24	4.72	-	2.865	10.64
2	10.0	15.54	1.34	4.55	-	2.497	10.38
2		8.52	3.18	2.94	-	0.880	13.05
2	0**	6.48	4.14	3.22	-	0.733	13.40
2	0**	11.50	2.13	1.82	-	0.740	12.22
4	0**	9.50	2.72	1.92	-	0.642	12.92

*=Control period before cholestyramine begun.

**=Recovery period after cholestyramine withdrawn.

TABLE VIII

STOOL COMPOSITION-LITTER 2

Rat	Diet Choles. %	Wt. of Stool (g/d)	Calcium % (by wt.)	Fat % (by wt.)	Soaps (mEq/d)	Total Fat (mEq/d)	Calcium mEq/d
5	0*	8.31	2.04	-	-	-	8.48
5	0*	9.58	2.12	2.01	-	0.676	10.16
6	0*	12.05	1.72	-	-	-	10.34
6	0*	9.65	2.06	1.73	0.093	0.586	9.88
7	0*	16.37	1.28	-	-	-	10.49
7	0*	12.82	2.01	1.47	0.114	0.666	12.88
8	0*	13.10	1.65	-	-	-	10.78
8	0*	11.70	1.93	1.73	-	0.714	11.28
5	2.5	12.50	2.01	3.82	-	1.678	12.54
5	2.5	12.60	2.15	3.54	0.343	1.568	13.54
6	2.5	12.50	2.02	3.16	-	1.389	12.66
6	2.5	13.35	2.48	2.75	0.146	1.293	16.54
7	5.0	15.45	1.92	4.21	-	2.291	14.81
7	5.0	18.05	1.87	3.80	0.909	2.460	16.80
8	5.0	15.20	1.69	6.06	-	3.251	12.80
8	5.0	17.60	1.47	4.07	-	2.253	12.98
8	0**	11.70	2.82	2.36	-	0.973	16.50

*=Control period before cholestyramine begun.

**=Recovery period after cholestyramine withdrawn.

FIG. 2 RELATIONSHIP BETWEEN
CHOLESTYRAMINE INTAKE AND STEATORRHEA

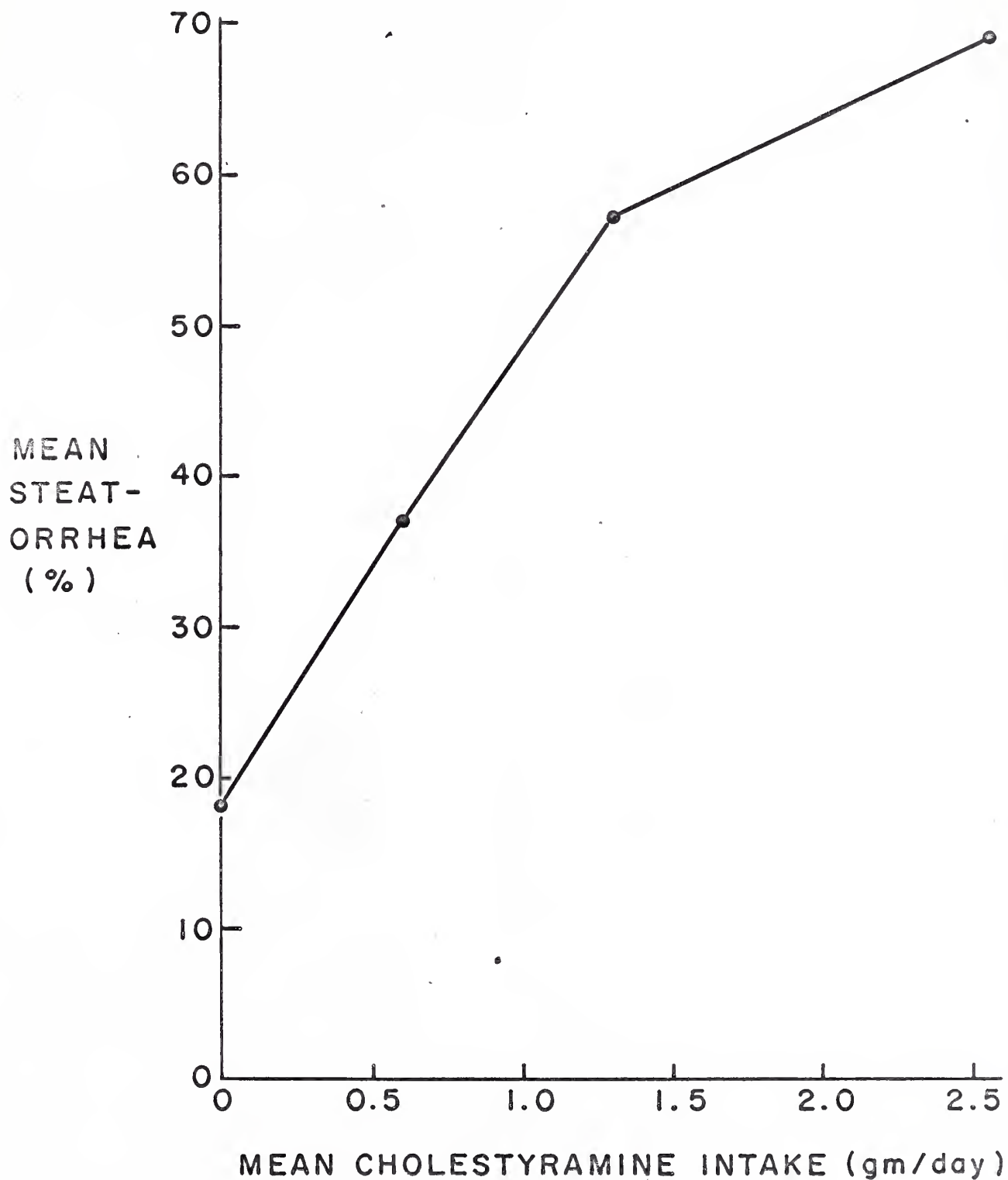


FIG. 3 RELATIONSHIP BETWEEN
STEATORRHEA AND FECAL SOAPS

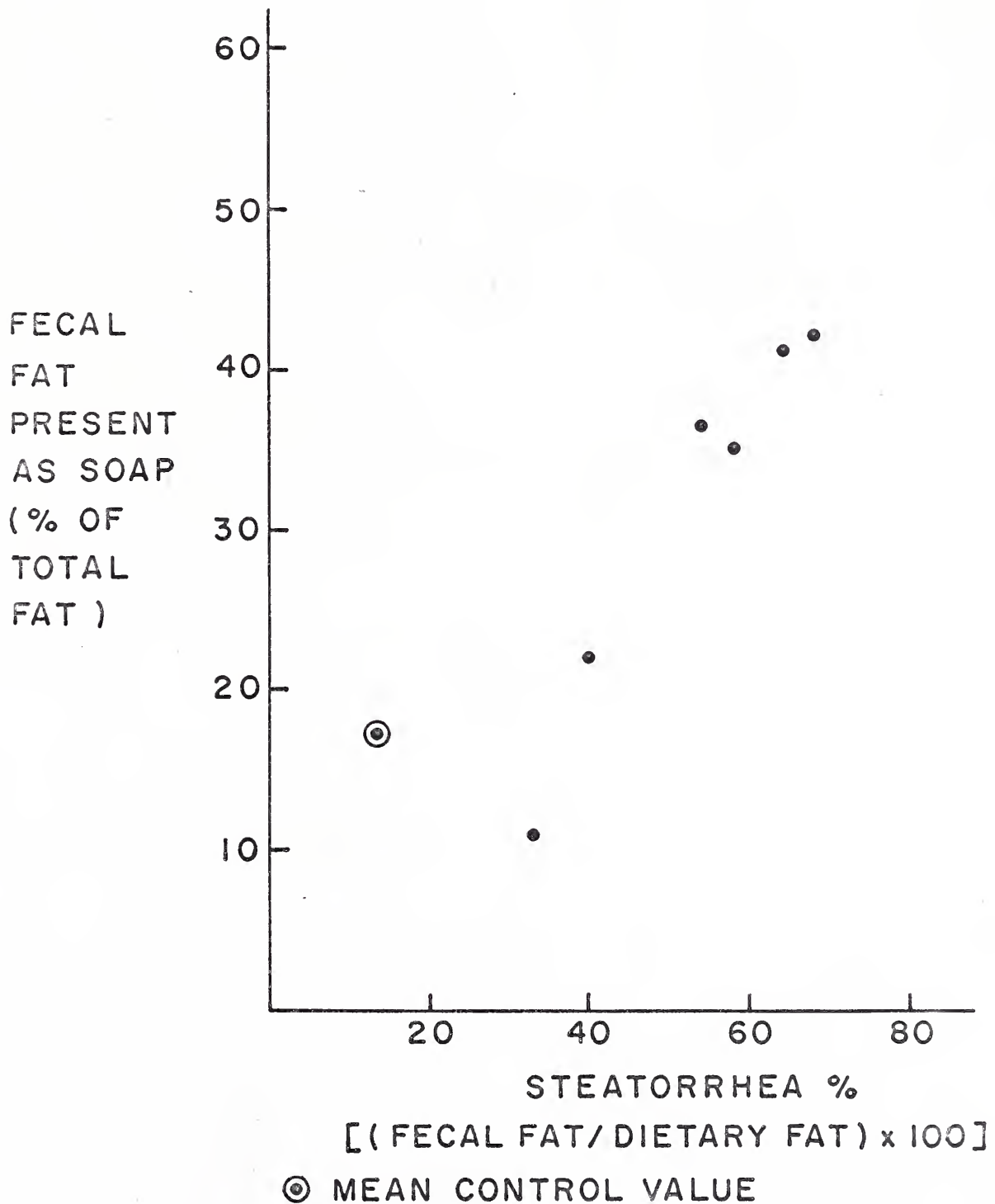


FIG. 4 RAT I

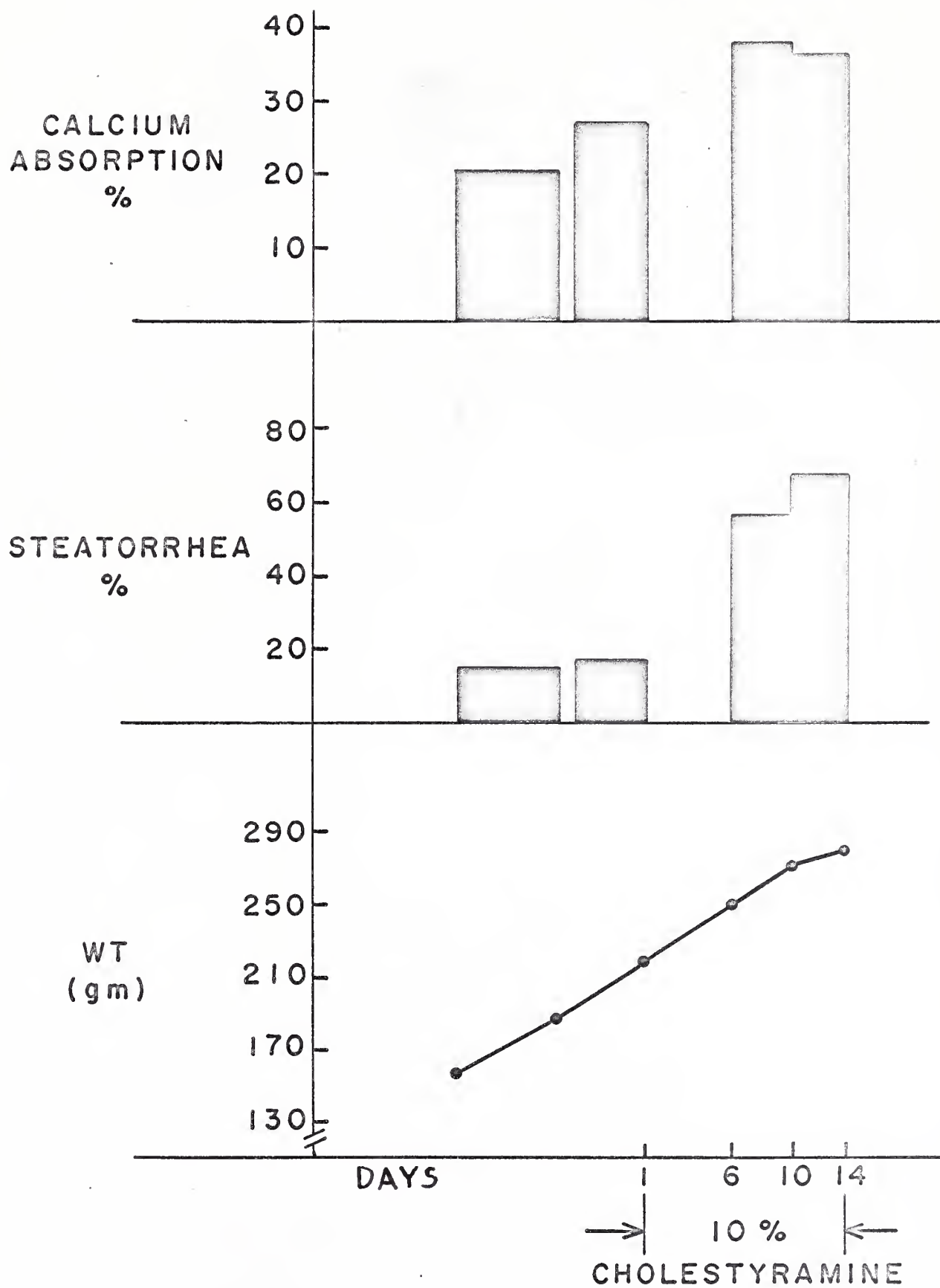


FIG. 5 RAT 2

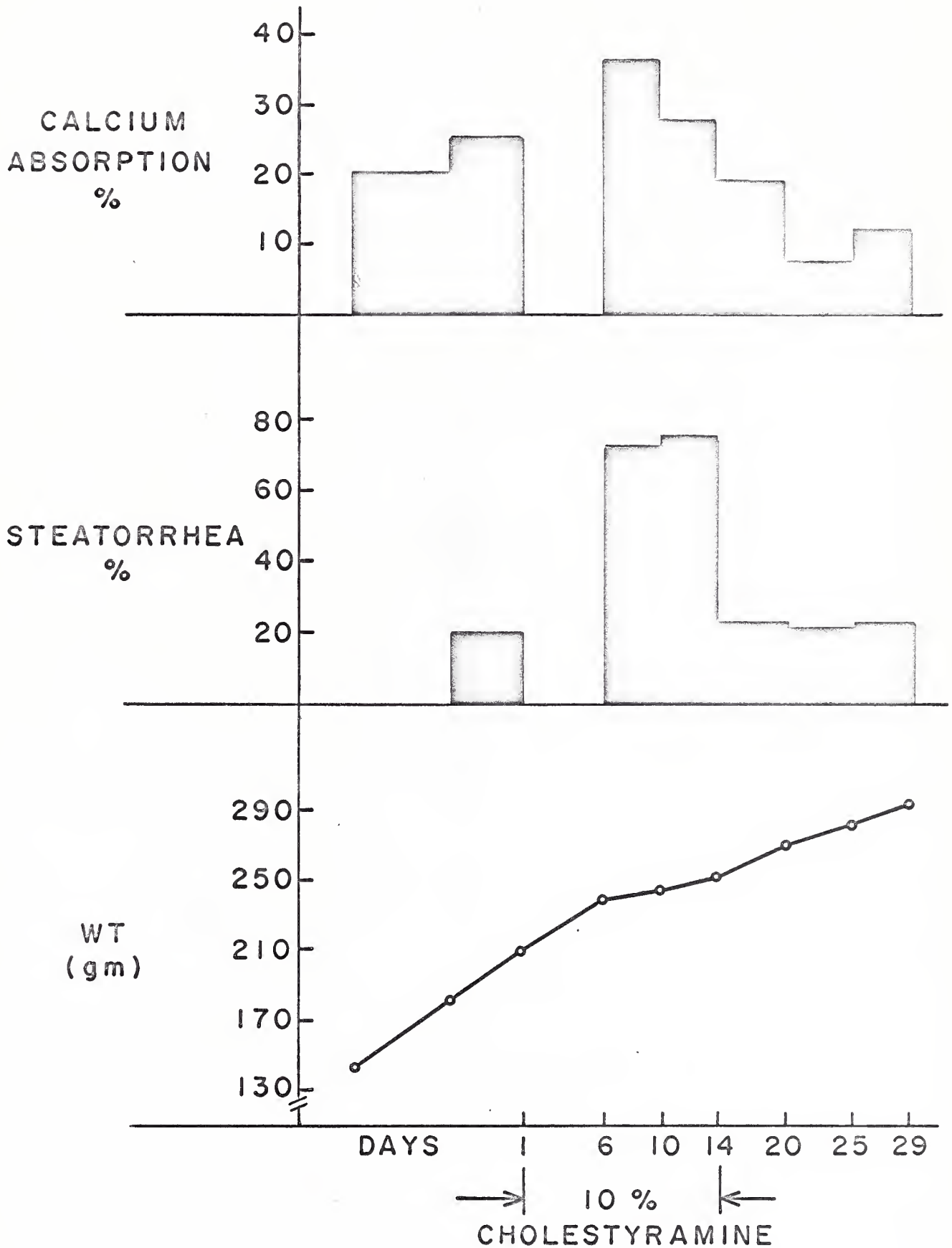


FIG. 6 RAT 3

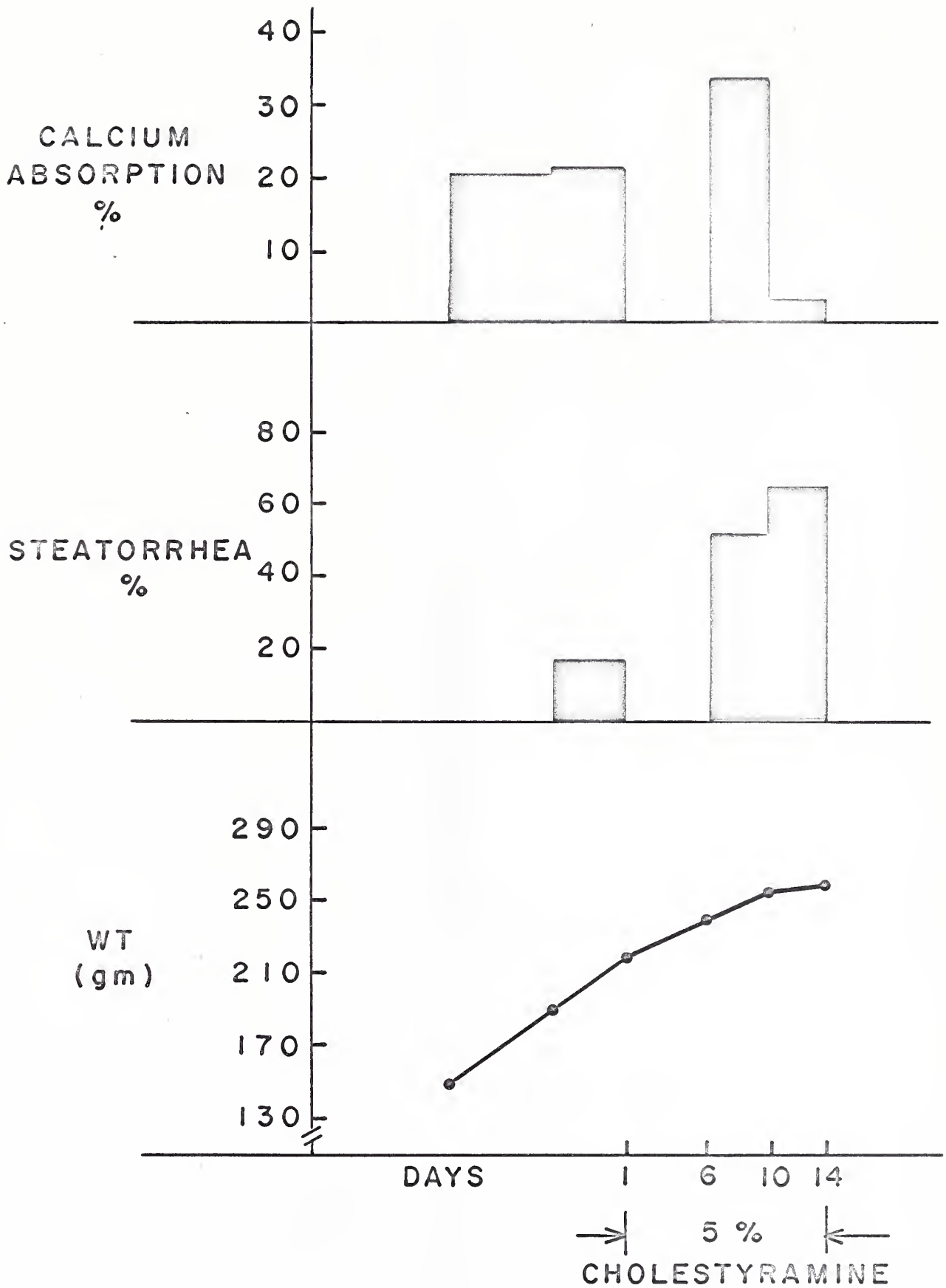


FIG. 7 RAT 4

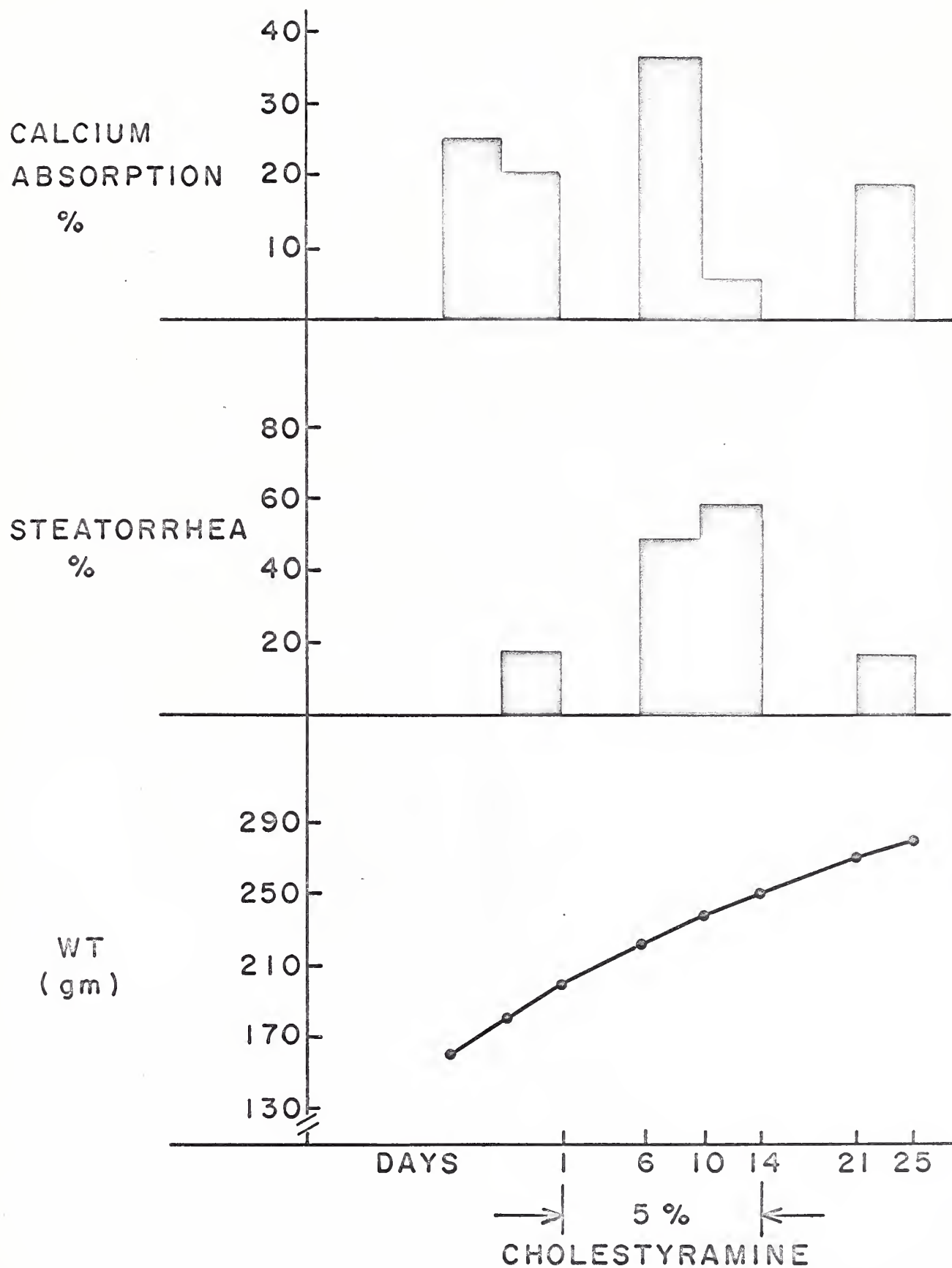


FIG. 8 RAT 5

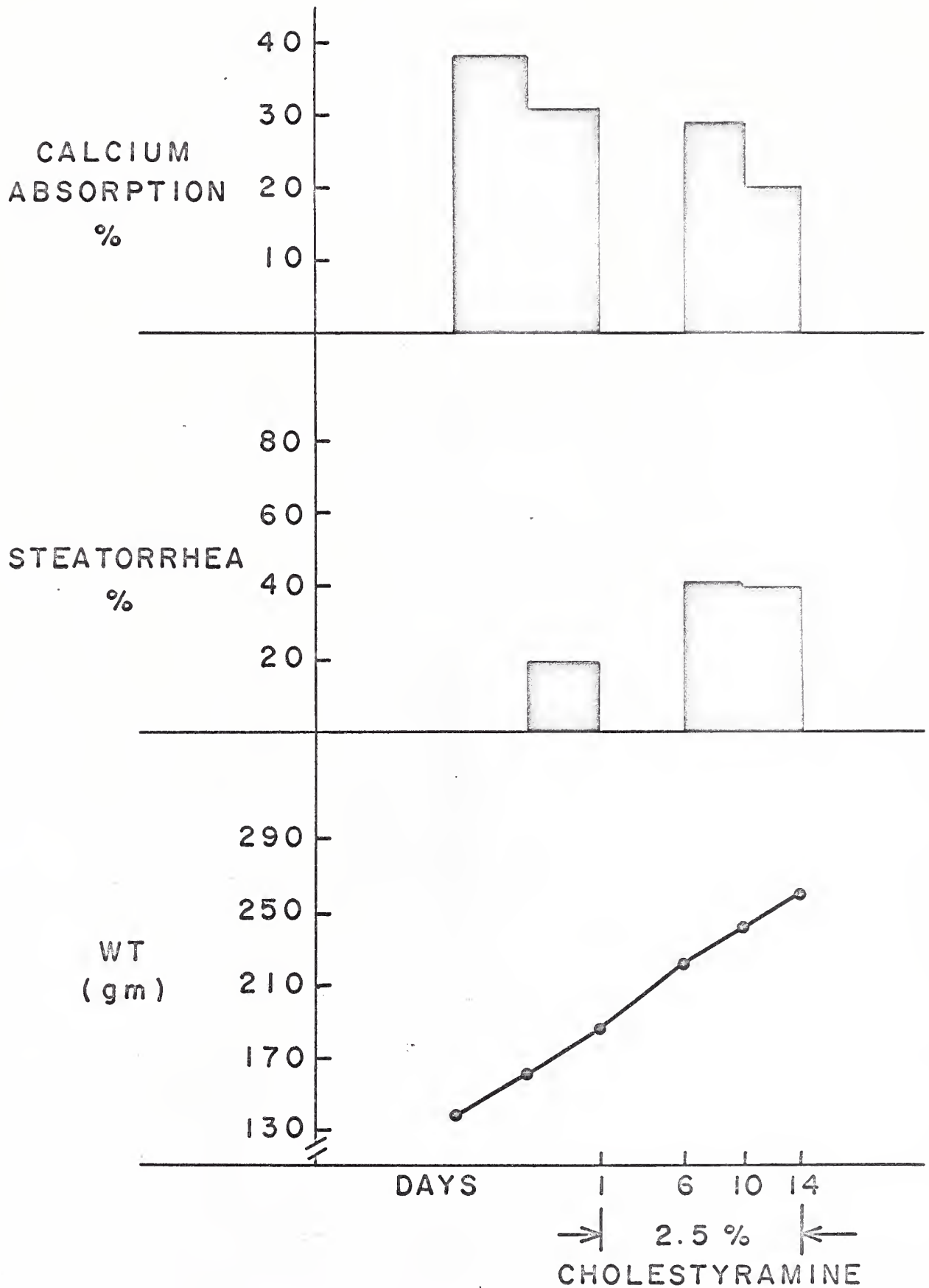


FIG. 9 RAT 6

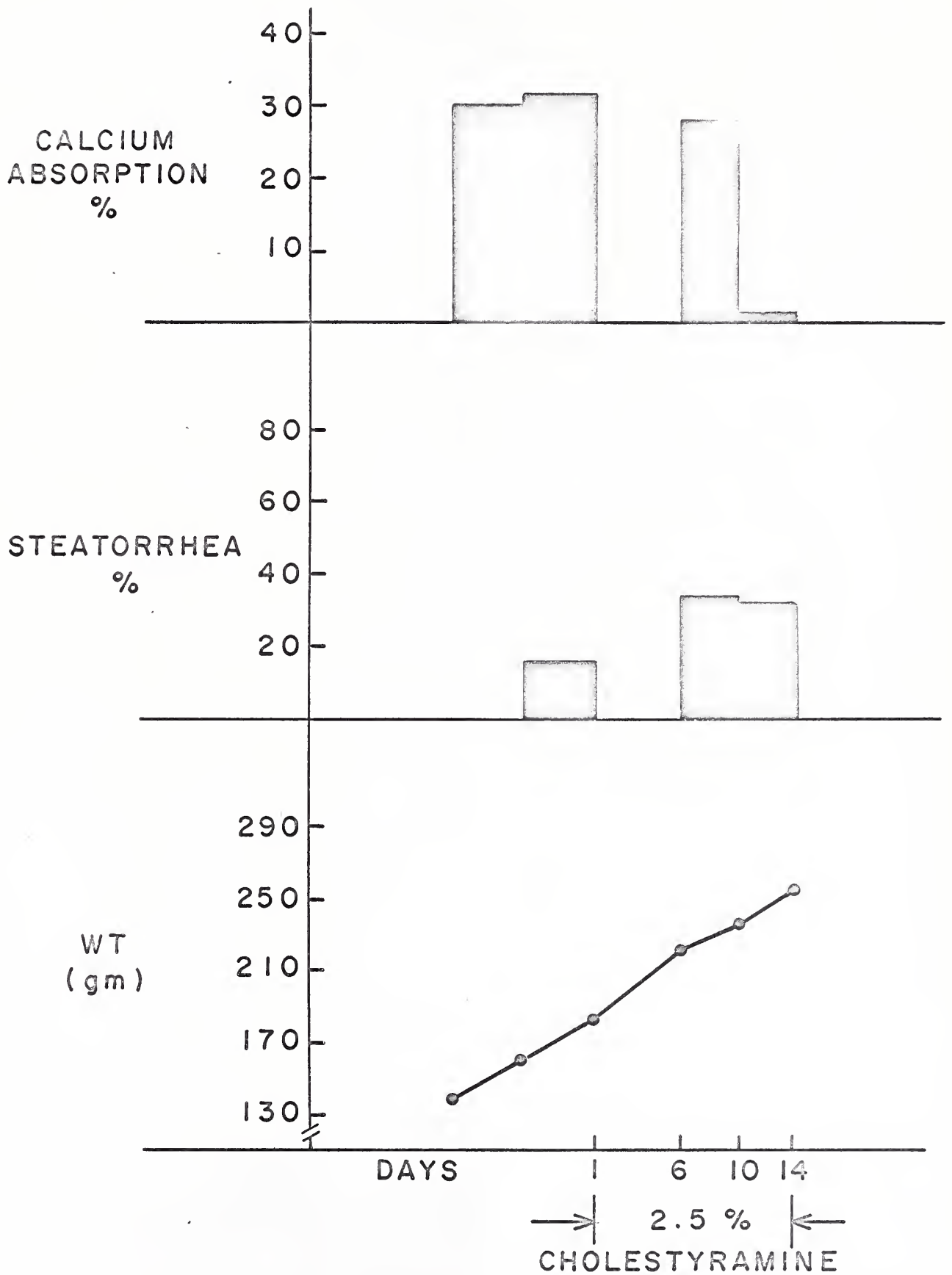


FIG. 10 RAT 7

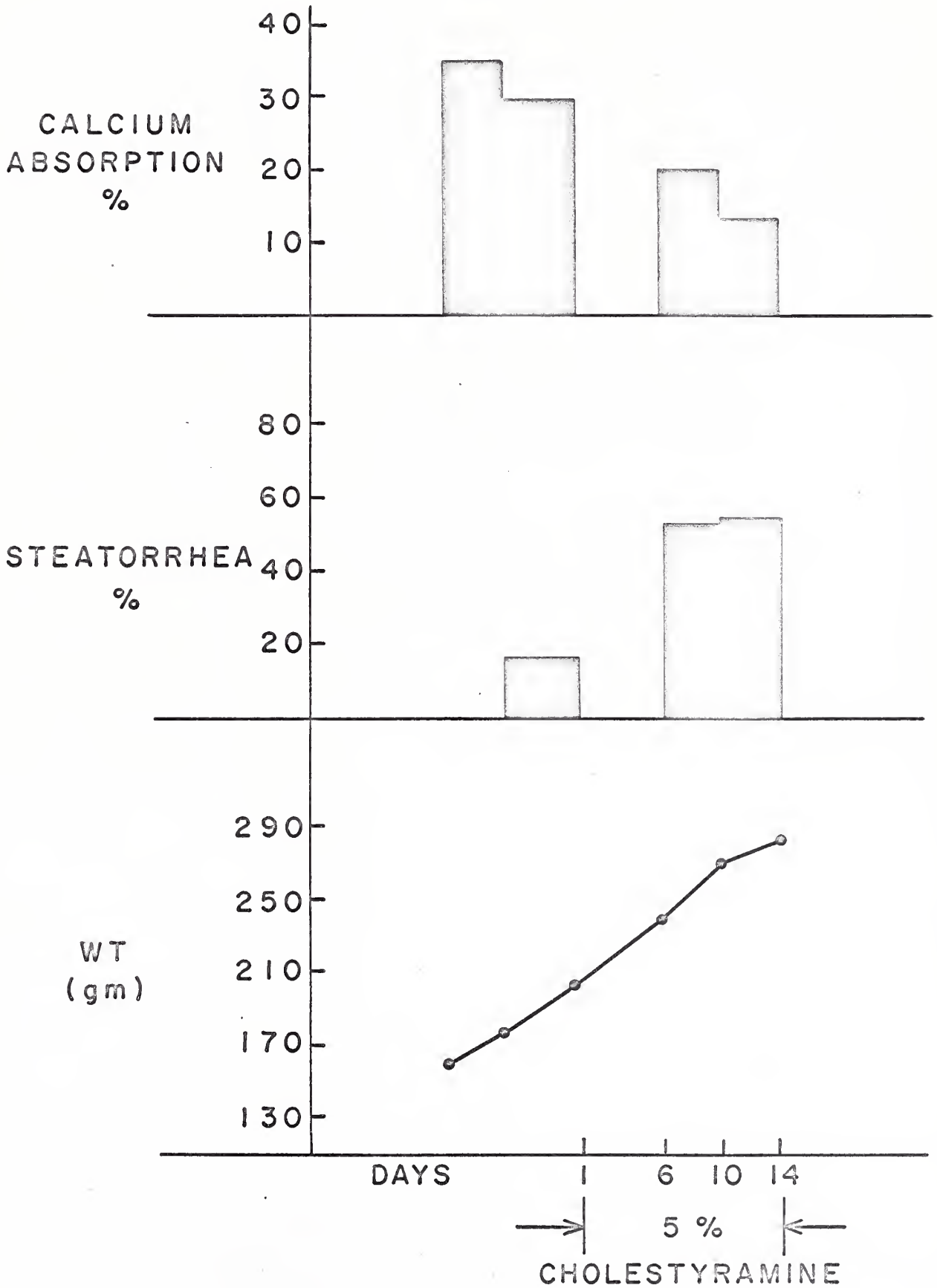


FIG. II RAT 8

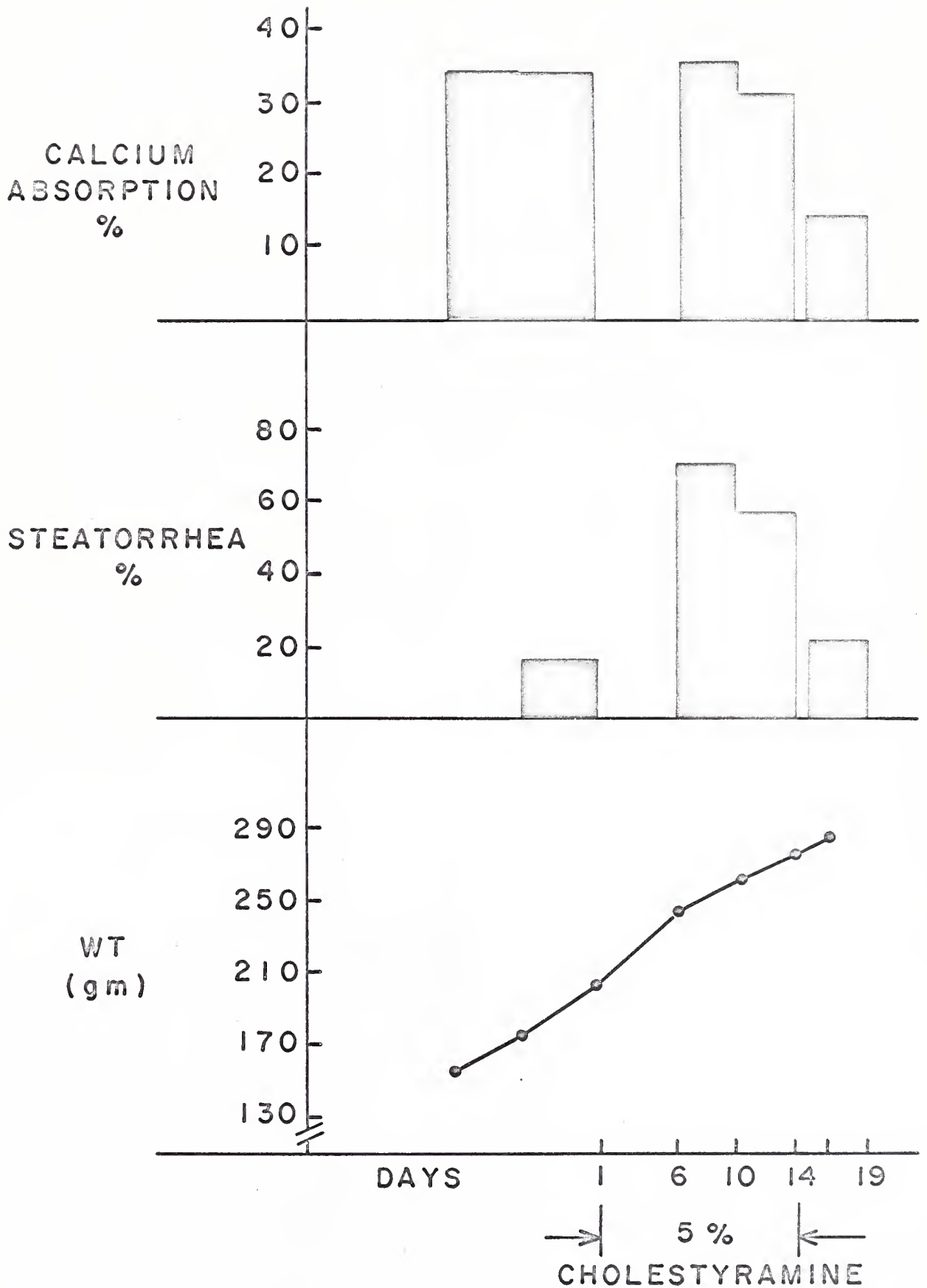
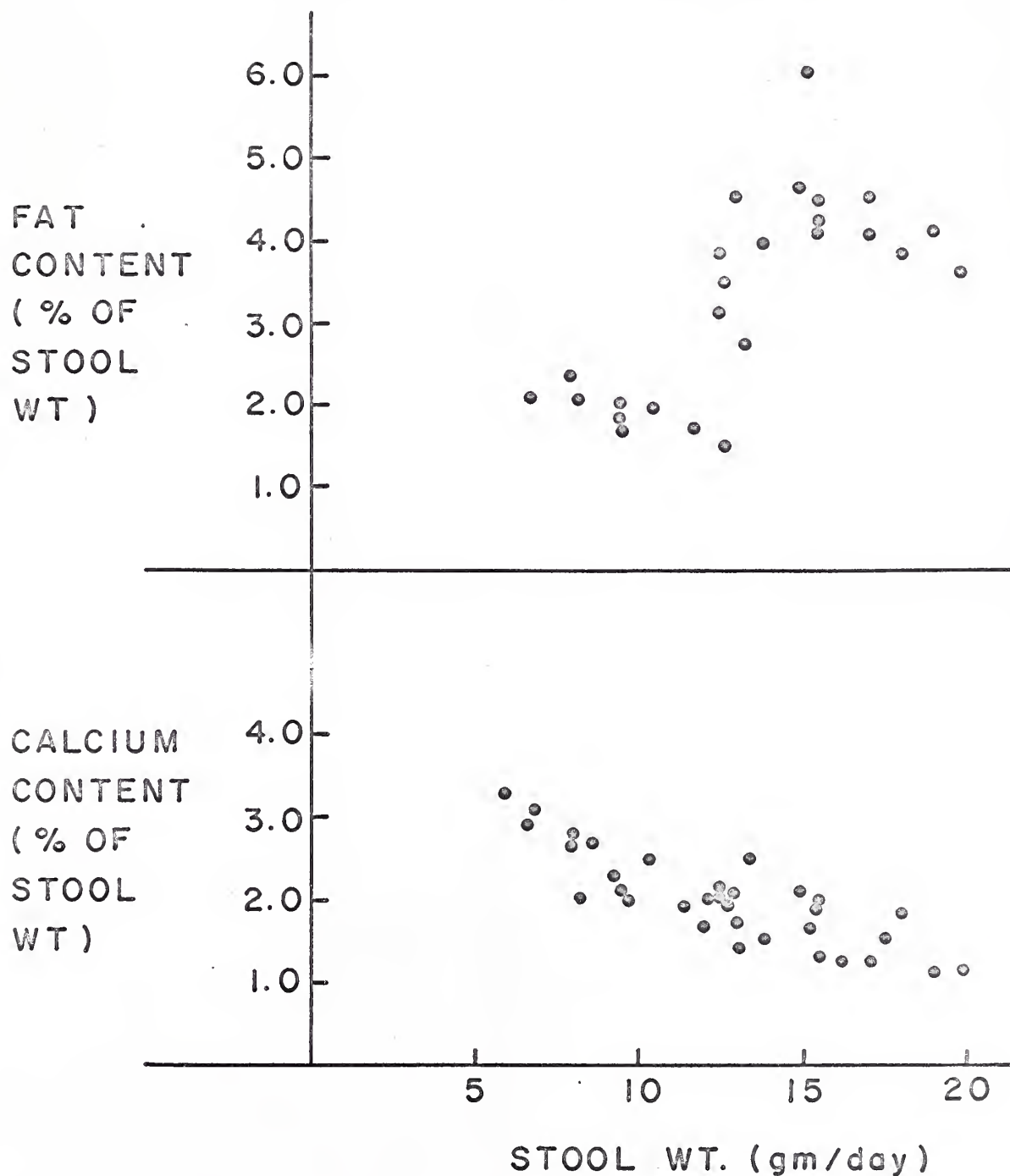


FIG. 12 RELATIONSHIPS BETWEEN
STOOL WEIGHTS AND CALCIUM
CONTENT AND FAT CONTENT



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